FEARNA STORAGE

Fearna Pumped Storage Hydro Scheme
EIA Report

Appendix 11.1.1 – eDNA Survey Report

February 2025

Quality Information

Prepared by	Checked by	Approved by
Gavia	Chris Pasteur	Fraser Allison

Revision History

Revision	Revision Date	Details	Authorised
01	February 2025	Issue to ECU	Fraser Allison

Appendix 11.1.1

Detection of Arctic charr (*Salvelinus alpinus*) utilising eDNA, molecular approaches and metabarcoding to determine the presence or absence in Scottish loch systems.

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Introduction

Environmental DNA (eDNA) coupled with metabarcoding is a developing molecular approach for species identification in environmental samples, i.e., soil, sediment, water etc, containing cellular and extracellular DNA shed off organisms. The methodology has been successfully employed within the Llewellyn lab and across the literature to detect, monitor, and estimate communities and populations of species in a particular environmental setting of interest. In this study, Arctic charr (*Salvelinus alpinus*) is of interest due to now being categorized as vulnerable in Britain and determining if the species can be detected in two closely located and connected loch systems in Scotland.

Therefore, Gavia Environmental have supplied 11 samples from the two loch systems in Scotland (Loch Quoich [n = 5] and Loch Fearna [n = 6] to undertake a trial to establish whether we can provide eDNA testing for the presence/absence of *Salvelinus alpinus* within the loch [lake] and river system. Using specific eDNA primers and qPCR protocol for *S. alpinus*, we aim to optimise this molecular approach to complete the following aims:

1. Determine the presence or absence of *S. alpinus* in Loch Quoich. Loch is known to contain *S. alpinus*.
2. Determine the presence or absence of *S. alpinus* in Loch Fearna. Loch is known to be free from *S. alpinus*.

Furthermore, using MiFish universal metabarcoding primers, we aim to:

1. Utilise eDNA metabarcoding of the MiFish locus for Loch Fearna samples (n = 6) to determine true presence of *S. alpinus*.

Methodology

Water sampling for eDNA collection

Conducted on-site by Toni Dwyer (Gavia Environmental) using standardized protocols developed in the Llewellyn lab using barrel filters. eDNA barrel filter samples stored at 4°C until extraction.

eDNA extraction and purification

The initial eDNA extraction process was conducted following the Llewellyn lab eDNA extraction protocol with no modifications (can be supplied if required). Further, eDNA extraction and purification were conducted using Qiagen DNeasy Blood and Tissue Kit (Qiagen, Doncaster, VIC, Australia) following the manufacturer's protocol.

Determination of extracted eDNA concentration

After extraction and purification, DNA concentration was determined using a Qubit 4.0 Fluorometer (Invitrogen, Waltham, MA, USA).

Primers for eDNA qPCR detection of *S. alpinus*

qPCR assay amplified a 164bp region of *S. alpinus* Cytochrome c oxidase subunit I gene using SAAL_CO1F and SAAL_CO1R primers (Integrated DNA Technologies, Leuven, Belgium) (Table 1).

Table 1: ***qPCR primers used for amplification of CO1 for the detection of S. alpinus.***

Protocol	Gene	Target	Primer	Name	Sequence (5' – 3')	Product size (bp)	Reference
qPCR	CO1	*S. alpinus*	Forward	SAAL_CO1F	CTTATAGTCATACCAATTATGATCGGG	164	Hernandez, *et al*., 2020
			Reverse	SAAL_CO1R	GCCAGCTCAACCCT		

qPCR detection of *S. alpinus*

Real-time PCR (qPCR) assay was conducted using Luna Universal qPCR Master Mix (New England BioLabs®, Ipswich, MA, USA). Each reaction (20µl) contained 2µl of extracted eDNA sample and 18µl of master mix which included: 650µl of Luna Universal qPCR Master Mix, 390µl nuclease-free water and 65µl of each primer SAAL_CO1F and SAAL_CO1R. All samples were assayed in triplicate including non-template controls and a 10-fold dilution series of 34.6ng/µl of extracted *S. alpinus* DNA (obtained from Sam Fenton [University of Glasgow]. Location - Loch Ard Achadh, 55.60416, -6.26805, Year - 2021). The reaction was conducted using an Aligent AriaMx Real-Time PCR System (Aligent Technologies, TX, USA). Initially, samples were incubated at 95°C for 20s followed by 40 cycles of 95°C for 3s, 60°C for 30s (Hernandez, *et al*., 2020) and a melt step for 1 cycle at 95°C for 30s, 65°C for 30s and 95°C for 30s.

MiFish primers for eDNA detection and metabarcoding of *S. alpinus*

PCR assay amplified an approx. 170bp fragment (will display larger on gel electrophoresis due to the addition of sequencing tags) from the mitochondrial 12S rRNA gene using MiFish_U_F-tag and MiFish_U_R-tag primers (Integrated DNA Technologies, Leuven, Belgium) (Table 2).

Table 2: ***MiFish primers used for amplification and sequencing of 12S rRNA for detection of S. alpinus.***

Protocol	Gene	Target	Primer	Name	Sequence (5' – 3')	Product size (bp)
PCR	12S rRNA	*S. alpinus*	Forward	MiFish_U_F-tag	ACA CTC T C TAC ACG ACG CTC TC CGA TCT GTC GT AAA ACT CGT GC AGC	Approx. 170
			Reverse	MiFish_U_R-tag	GTG ACT GGA GT CAG ACG TGT GCT CT CG ATC TCA TAG TG GT ATC TAA TC CAG T G	

PCR detection of MiFish locus of *S. alpinus*

12S rRNA test detection utilising MiFish universal primers

PCR assay was conducted using Q5 High fidelity (New England BioLabs®, Ipswich, MA, USA) and ToughMix (QuantaBio, Beverly, MA, USA) PCR Master Mix(s). Each reaction (20µl) contained 1µl of extracted DNA and 19µl of master mix which included: 300µl (Q5 or toughmix), 210µl 390µl nuclease-free water and 20µl of each primer MiFish_U_F-tag and MiFish_U_R-tag. All samples were assayed in singular using both PCR master mixes including non-template control and 34.6ng/µl of extracted *S. alpinus* DNA. The reaction was conducted using a Primer Thermal Cycler (Cole-Parmer LTD, . Initially,

samples were incubated at 94°C for 5 minutes, followed by 35 cycles of 98°C for 20s, 65°C for 15s, 72°C for 15s, with a final elongation step of 72°C for 5 minutes. Samples were run on a 1% agarose gel at 75v for 90 minutes.

MiFish metabarcoding

Based on results from MiFIsh test detection, samples (n=6) were assayed using Toughmix with all other steps remaining the same. Samples were then Nanodropped and diluted to concentrations between 43 – 95ng/µl. A bead clean-up was then conducted before sequencing through Illumina.

Visualisation and statistical analysis

qPCR results were obtained and visualised as an amplification plot and standard curve within AriaMx software version 1.8. Statistical analysis was conducted using R version 4.1.2 and RStudio version 21.09.1 Build 372.

Initial, MiFish test results were visualised through gel electrophoresis to ensure amplification. Metabarcoding was conducted

Results

DNA concentrations of extracted samples

Table 3: ***DNA concentrations of +ve control and extracted eDNA samples****. [<LOD =Below the Limit of Detection].*

Sample ID	Sample Type	Location	Concentration (ng/µl)
ELT12886	+ ve	Loch Ard Achadh	34.6
QLFT1	eDNA	Loch Quoich	11
QUP1	eDNA	Loch Quoich	8.44
QUP2	eDNA	Loch Quoich	9.72
Q2R	eDNA	Loch Quoich	6.92
QBRS	eDNA	Loch Quoich	11.2
FSL1	eDNA	Loch Fearna	13.7
FSL3	eDNA	Loch Fearna	5.38
FSLX3	eDNA	Loch Fearna	<LOD
FSL4	eDNA	Loch Fearna	13.2
FSL5	eDNA	Loch Fearna	8.06
FSL5B	eDNA	Loch Fearna	5.90

Specificity of qPCR assay

The sensitivity of the qPCR assay was estimated by a 10-fold dilution series of 34.6ng/µl from 1/10 to 1/10,000,000 to calibrate a standard curve of abundance (ng/µl) for unknown eDNA samples. Co-efficient of determination (R^2 = 0.992) with an efficiency of 102.56% (Figure 1).

y = -3.262x + 17.48 R^2 = 0.992 Efficiency = 102.56%

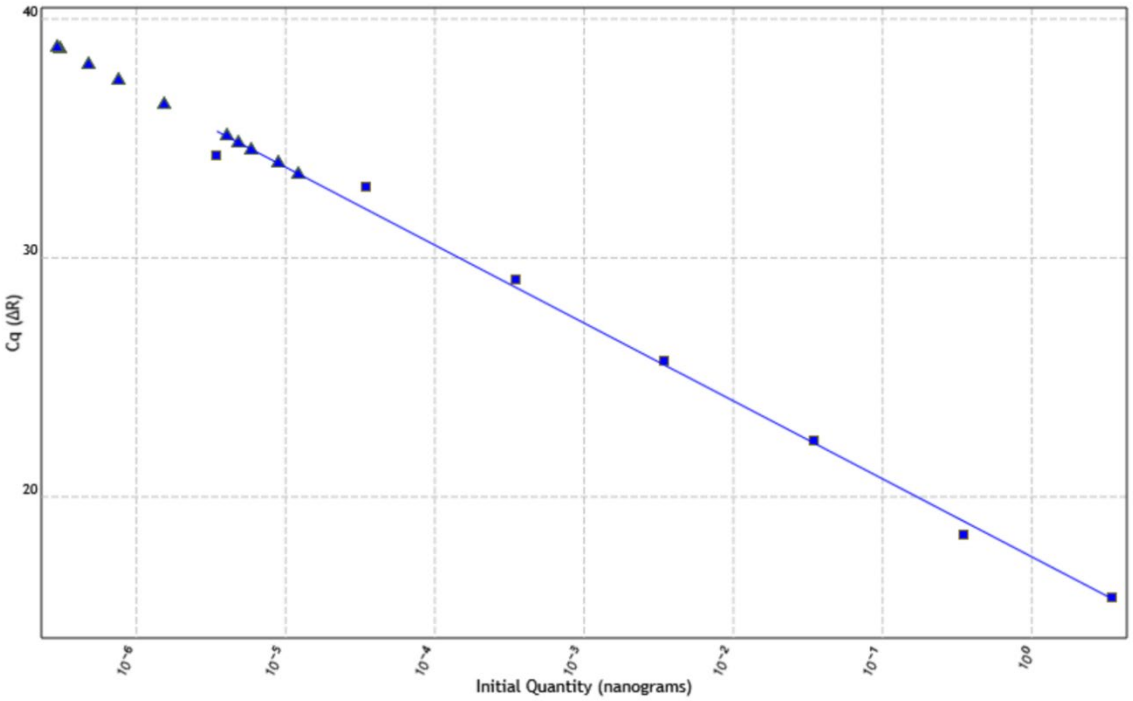

Figure 1: ***Standard curve of +ve including the abundance of S. alpinus in water samples.*** *Triangles represent the average Cq of unknown samples and squares indicate the average Cq of known concentrations. Non-template controls are excluded from the standard curve.*

Determination of the presence or absence of *S. alpinus* in the marine environment

The presence or absence of *S. alpinus* was determined for each loch (Quoich & Fearna) by qPCR assay against a positive extraction of *S. alpinus* from Loch Ard Achadh (Table 3). All values are displayed in triplicate per sample location (Results in triplicate can be seen in Appendix 6). Due to the low nanogram values detected through the qPCR assay we can determine that detection of *S. alpinus* in the two loch systems is not possible and <LOD. An ANOVA indicates no significant difference between site and negative controls ($F\text{-value}_{2,33}$ = 0.035, p-value = 0.965). Further analysis using a TukeyHSD further revealed no statistical significance between lochs or negative control.

- Loch Quoich – Loch Fearna, p-value = 0.969
- Negative control – Loch Fearna, p-value = 0.984
- Negative control – Loch Quoich, p-value = 0.999

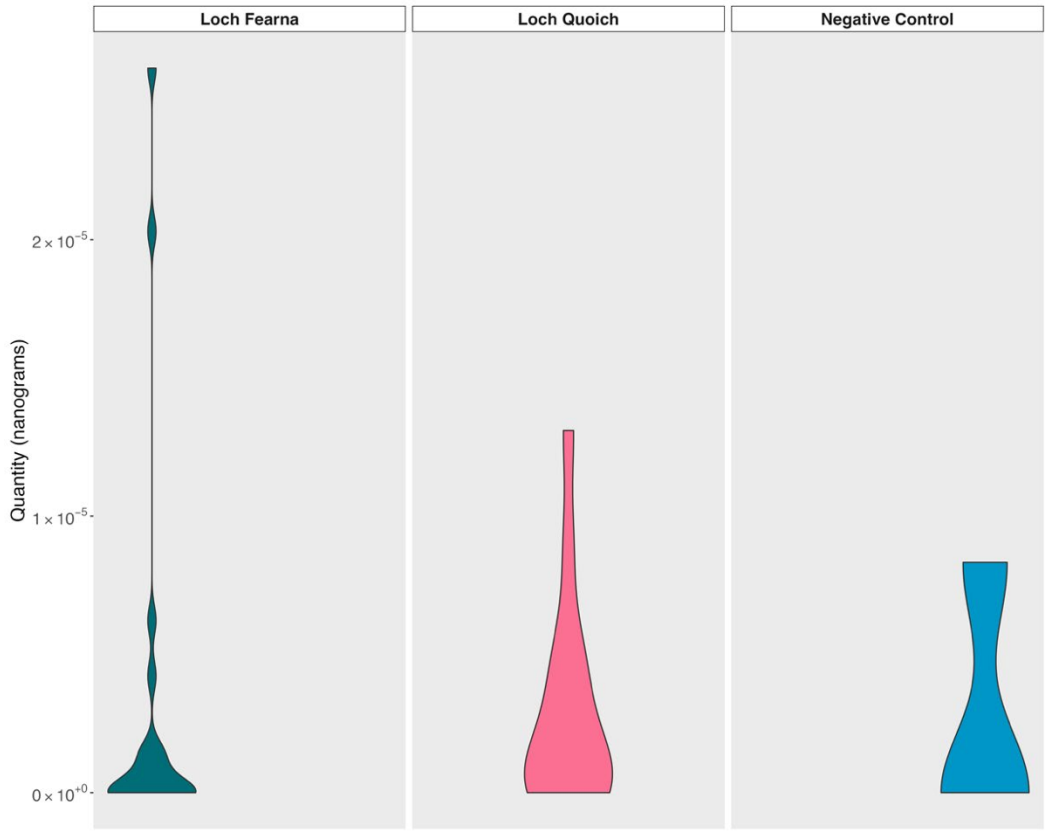

Figure 2: ***Quantity of eDNA amplification of CO1 gene in S. alpinus across site and negative control.***

PCR detection of 12S rRNA MiFish loci

To determine whether the 12S rRNA gene was represented within water column eDNA samples, MiFish universal primers were utilised. Visualisation of the PCR product yielded a positive detection of the 12S rRNA gene through gel electrophoresis with distant and clear banding at roughly 250bp (larger due to sequencing tags)(Figure 3). Positive control of *S. alpinus* displayed the correct size of fragment with Non-template controls revealing no banding as expected.

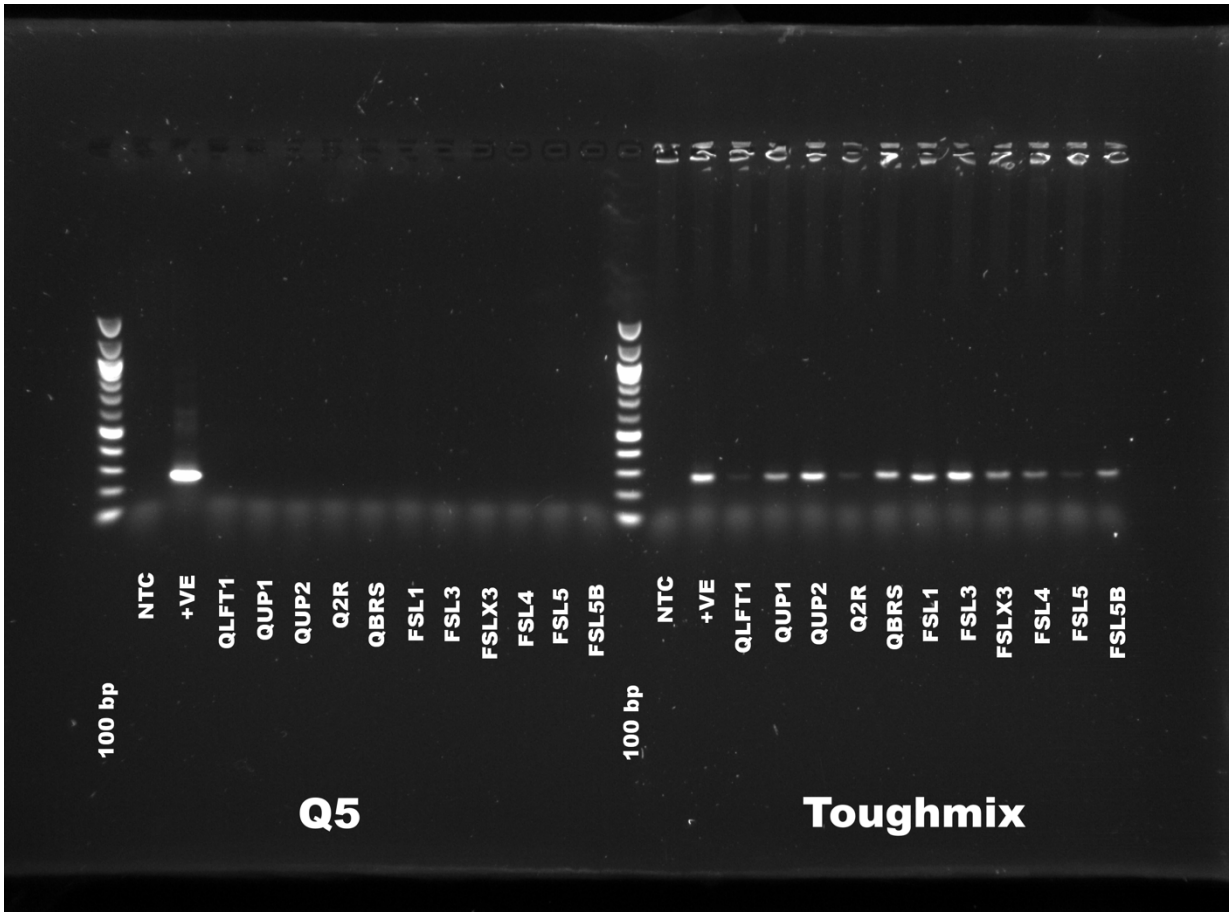

Figure 3: ***Amplification of 12S rRNA gene through PCR and expressed using gel electrophoresis.*** *PCR amplified 12S rRNA gene for eDNA samples and displayed banding at the correct size when using toughmix PCR mastermix. No banding was seen when using NEB Q5 High-fidelity PCR mastermix.*

Read/Species Statistics from MiFish Sequencing

Table 4: ***Read statistics of MiFish metabarcoding data.***

Site	Read type	No. Reads	No. Reads after Quality Filter	No. Assembled Reads	No. Reads without N	No. Reads Fit Length	No. Unique Reads	No. Denoised Haploids	No. Species
FSL1	Pair-End FastQ	65,004	65,004	64,387	64,374	62,871	3,462	3	3
FSL3	Pair-End FastQ	64,400	64,400	64,393	64,385	64,137	2,895	1	1
FSLX3	Pair-End FastQ	64,632	64,632	64,614	64,591	64,374	2,698	1	1
FSL4	Pair-End FastQ	63,182	63,182	63,156	63,137	62,756	2,637	2	1
FSL5	Pair-End FastQ	62,514	62,514	62,350	62,338	61,958	2,979	4	2
FSL5B	Pair-End FastQ	61,552	61,552	61,537	61,522	61,286	2,707	3	2

Taxonomic Table from MiFish Sequencing

Site	Species	Total Reads	Confidence	Identity (%)	Confidence Score	Alignment Length	Mismatch
FSL1	*Salmo salar*	62,280	High	100	1.39	170	0
FSL1	*Homo sapiens*	285	High	100	1.61	171	0
FSL1	*Mallotus*	34	High	99.4	-	168	1
FSL3	*Salmo salar*	63,850	High	100	1.6	169	0
FSLX3	*Salmo salar*	64,130	High	100	1.39	170	0
FSL4	*Salmo salar*	62,458	High	100	1.6	169	0
FSL5	*Salmo salar*	49,326	High	100	1.6	169	0
FSL5	*Homo sapiens*	38	High	100	1.61	171	0
FSL5B	*Salmo salar*	60,216	High	100	1.39	170	0
FSL5B	*Sus celebensis*	32	Low	98.24	0.001	170	3

See the attached Excel spreadsheet for further details.

Relative Abundance of Genus from MiFish Sequencing

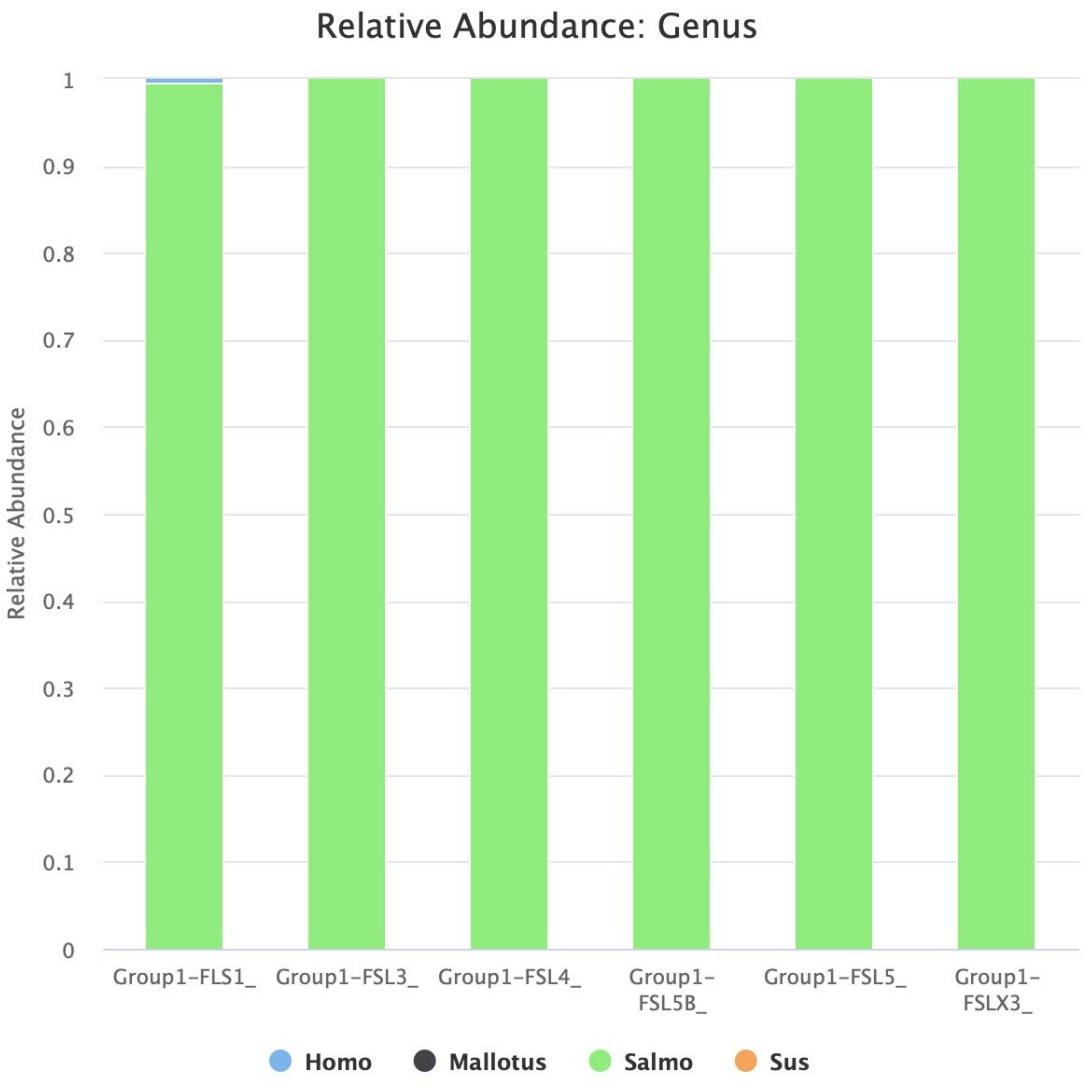

Figure 4: ***Relative abundance of genus across all eDNA sample sites of Loch Fearna.***

Conclusion(s)

- Presence or absence cannot be determined in either loch system due to being lower than the limit of detection/quantification as stated by the qPCR standard curve. Furthermore, both Loch samples were indistinguishable from the negative control.
- Melt-curve analysis (Appendix 3) indicates that Loch sample background amplicons are unlikely to be Charr in origin.
- To improve sensitivity and determine the presence or absence of *S. alpinus*, it is suggested an increase in water be pumped through the filter during eDNA sampling to improve the potential of a positive detection. Blocking of the filter could be avoided by coarse pre-filtration of the water sample prior to pumping.
- Metabarcoding of Loch Fearna samples revealed 12S rRNA to be a 100% match to *Salmo salar* (Atlantic salmon) and are unlikely to be *S. alpinus* (97.65% match when Blasted through NCBI).

Appendices

Appendix 1 – Amplification plot of qPCR Assay (Average)

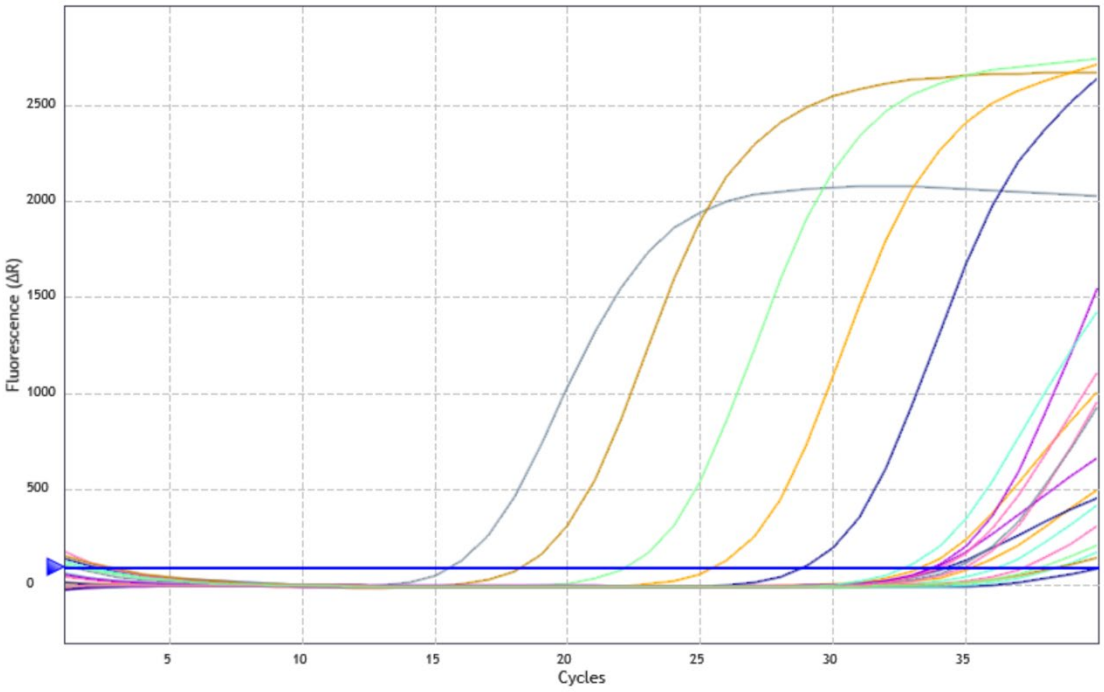

Figure 5: ***Averaged amplification of unknown eDNA samples and known concentrations of S. alpinus.*** *qPCR amplification of CO1 gene of S. alpinus. Amplification plot obtained through AriaMx qPCR software (Agilent Technologies, TX, USA). Sample size of unknown samples, n = 11 Known concentrations, n = 7.*

Appendix 2 – Amplification plot of qPCR Assay (Triplicate)

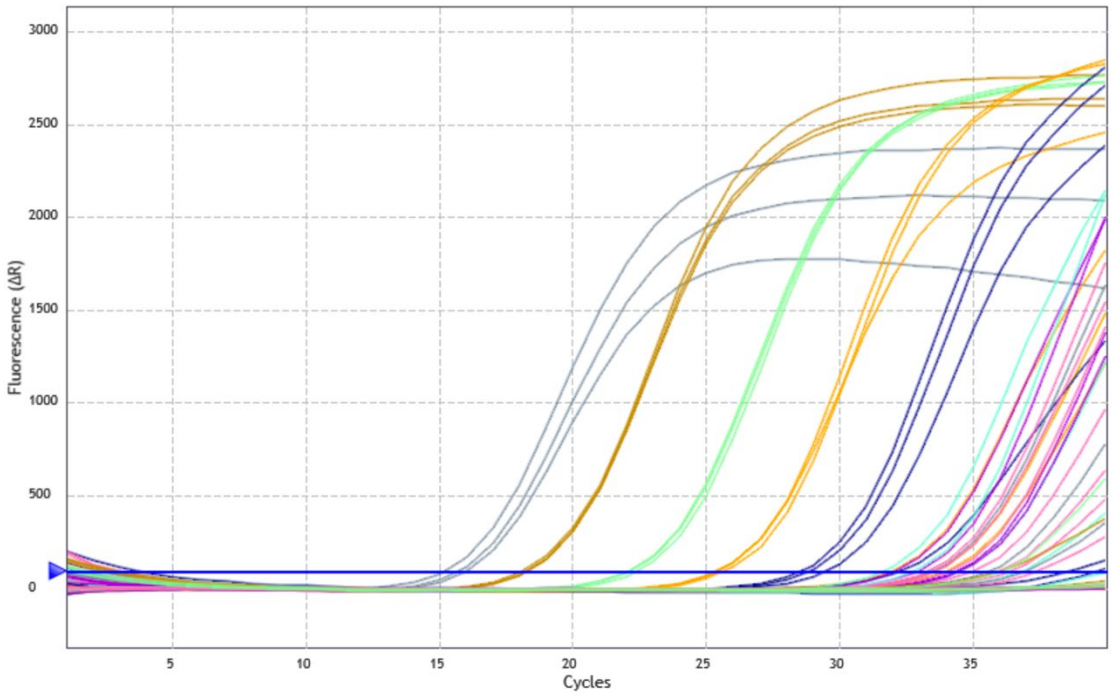

Figure 6: ***Triplicate amplification of unknown eDNA samples and known concentrations of S. alpinus.*** *qPCR amplification of CO1 gene of S. alpinus. Amplification plot obtained through AriaMx qPCR software (Agilent Technologies, TX, USA). Sample size of unknown samples, n = 11 (n = 33 in triplicate) Known concentrations, n = 7 (n = 21 in triplicate).*

Appendix 3 – Melt Raw Derivative Curve of qPCR Assay (Average)

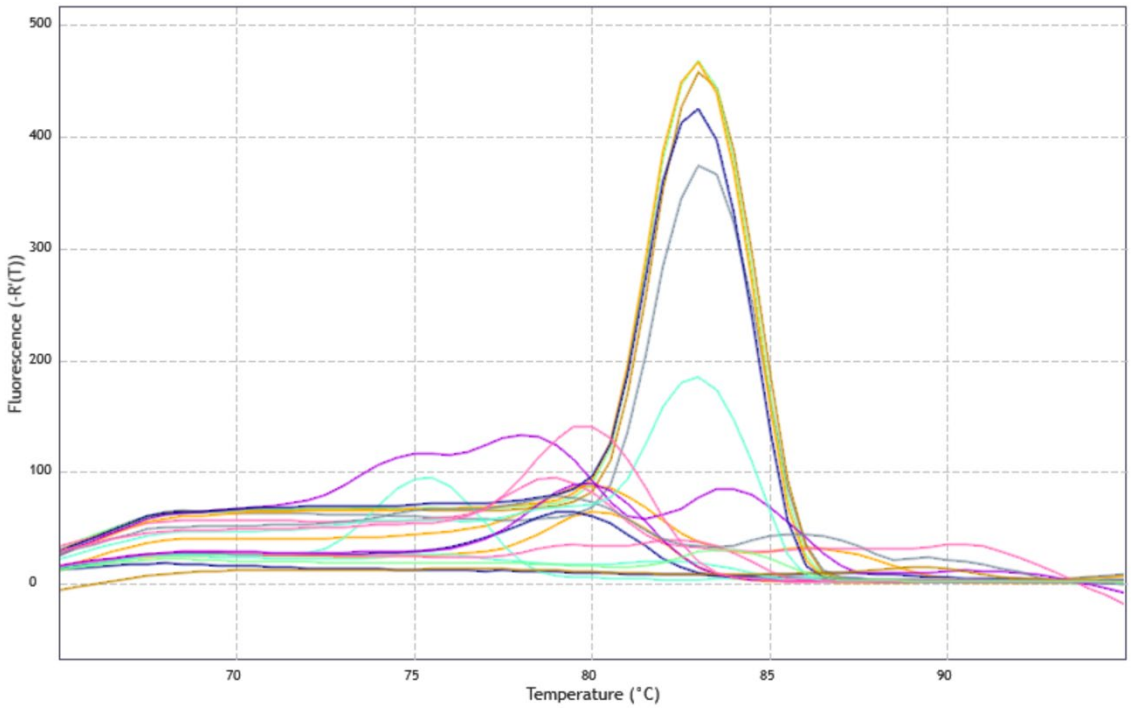

Figure 7: ***Melt Raw Derivative curve of qPCR assay.*** *Displayed as average fluorescence for each sample (n = 11), Standard (n = 7), and Negative controls (n = 2).*

Appendix 4 – Melt Raw Derivative Curve of qPCR Assay (Triplicate)

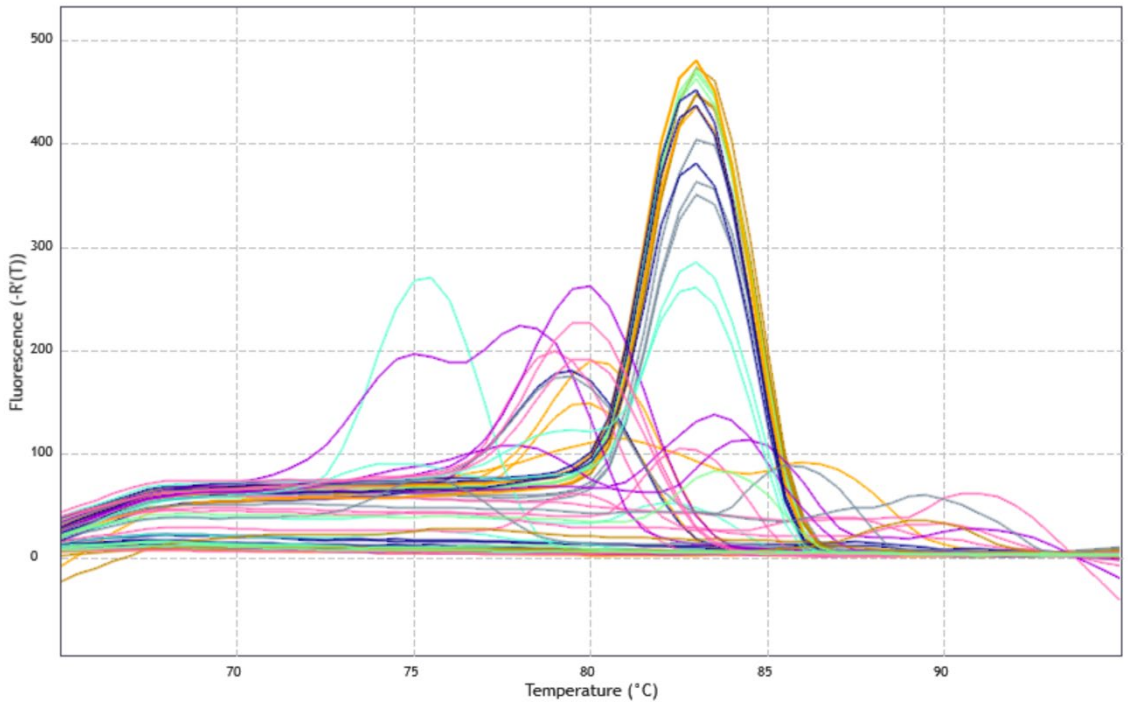

Figure 8: ***Melt Raw Derivative curve of qPCR assay.*** *Displayed as triplicate fluorescence for each sample (n = 33), Standard (n = 21), and Negative controls (n = 6).*

Appendix 5 – Standard Curve (Triplicate)

y = -3.19x + 17.53 R^2 = 0.989 Efficiency = 105.81%

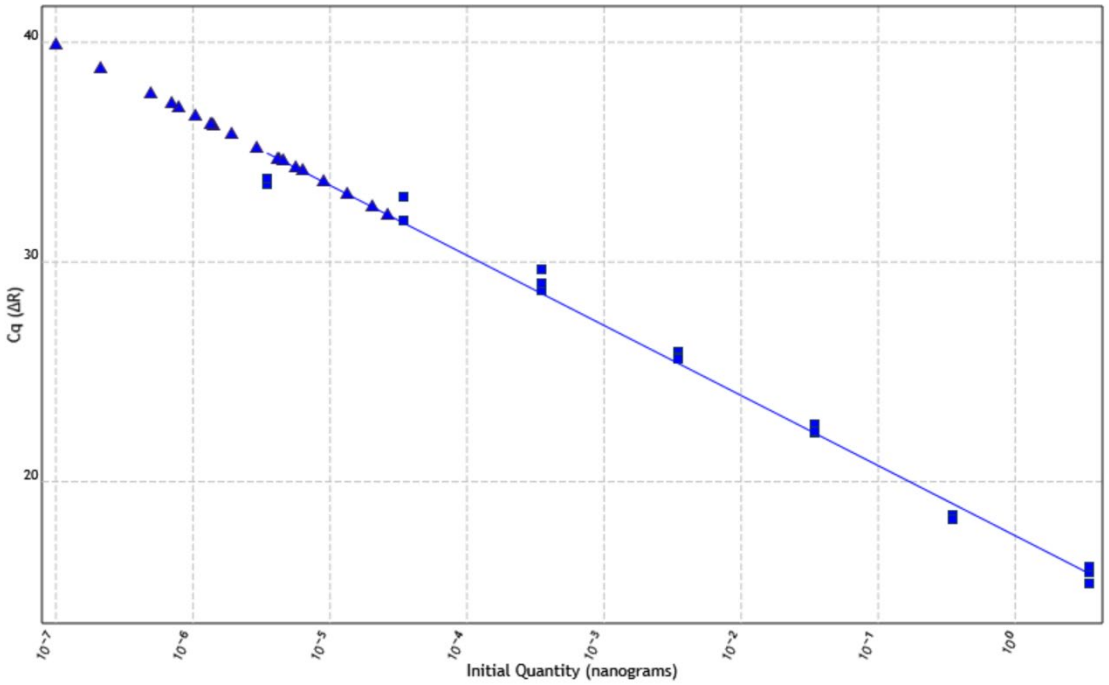

Figure 9: ***Standard curve in triplicate of +ve including the abundance of S. alpinus in water samples.*** *Triangles represent Cq of unknown samples and squares indicate Cq of known concentrations. Non-template controls are excluded from the standard curve.*

Appendix 6 – Results of qPCR Assay (Triplicate)

Table 3: ***Triplicate results of qPCR assay amplifying the CO1 gene of S. Alpinus.***

Sample ID	Replicate	Sample Type	Location	Cq	Quantity (ng)	Quantity Standard Deviation (ng)
NTC	1	Non-template control	N/A	33.73	$8.33x10^{-6}$	0
NTC	2	Non-template control	N/A	No Cq	0	0
NTC	3	Non-template control	N/A	No Cq	0	0
NTC	1	Non-template control	N/A	32.33	$2.3x10^{-5}$	0
NTC	2	Non-template control	N/A	No Cq	0	0
NTC	3	Non-template control	N/A	No Cq	0	0
1/10	1	Standard	Loch Ard Achadh	15.88	3.46	0
1/10	2	Standard	Loch Ard Achadh	16.13	3.46	0
1/10	3	Standard	Loch Ard Achadh	15.33	3.46	0
1/100	1	Standard	Loch Ard Achadh	18.29	$3.46x10^{-1}$	0
1/100	2	Standard	Loch Ard Achadh	18.50	$3.46x10^{-1}$	0
1/100	3	Standard	Loch Ard Achadh	18.46	$3.46x10^{-1}$	0
1/1000	1	Standard	Loch Ard Achadh	22.28	$3.45x10^{-2}$	0
1/1000	2	Standard	Loch Ard Achadh	22.57	$3.45x10^{-2}$	0
1/1000	3	Standard	Loch Ard Achadh	22.23	$3.45x10^{-2}$	0
1/10,000	1	Standard	Loch Ard Achadh	25.58	$3.46x10^{-3}$	0
1/10,000	2	Standard	Loch Ard Achadh	25.64	$3.46x10^{-3}$	0
1/10,000	3	Standard	Loch Ard Achadh	25.87	$3.46x10^{-3}$	0
1/100,000	1	Standard	Loch Ard Achadh	29.00	$3.46x10^{-4}$	0
1/100,000	2	Standard	Loch Ard Achadh	28.73	$3.46x10^{-4}$	0
1/100,000	3	Standard	Loch Ard Achadh	29.66	$3.46x10^{-4}$	0
1/1,000,000	1	Standard	Loch Ard Achadh	No Cq	0	0
1/1,000,000	2	Standard	Loch Ard Achadh	32.99	$3.46x10^{-5}$	0
1/1,000,000	3	Standard	Loch Ard Achadh	31.87	$3.46x10^{-5}$	0
1/10,000,000	1	Standard	Loch Ard Achadh	33.80	$3.46x10^{-6}$	0
1/10,000,000	2	Standard	Loch Ard Achadh	No Cq	0	0
1/10,000,000	3	Standard	Loch Ard Achadh	33.52	$3.46x10^{-6}$	0
QLFT1	1	eDNA	Loch Quoich	No Cq	0	0
QLFT1	2	eDNA	Loch Quoich	39.88	$9.90x10^{-8}$	$7.93x10^{-8}$
QLFT1	3	eDNA	Loch Quoich	38.83	$2.11x10^{-7}$	$7.93x10^{-8}$
QUP1	1	eDNA	Loch Quoich	No Cq	0	0
QUP1	2	eDNA	Loch Quoich	37.01	$7.84x10^{-7}$	0
QUP1	3	eDNA	Loch Quoich	No Cq	0	0
QUP2	1	eDNA	Loch Quoich	36.62	$1.03x10^{-6}$	$2.27x10^{-6}$
QUP2	2	eDNA	Loch Quoich	35.21	$2.87x10^{-6}$	$2.27x10^{-6}$
QUP2	3	eDNA	Loch Quoich	34.30	$5.55x10^{-6}$	$2.27x10^{-6}$
Q2R	1	eDNA	Loch Quoich	33.11	$1.31x10^{-5}$	$5.08x10^{-6}$
Q2R	2	eDNA	Loch Quoich	34.70	$4.14x10^{-6}$	$5.08x10^{-6}$
Q2R	3	eDNA	Loch Quoich	34.60	$4.45x10^{-6}$	$5.08x10^{-6}$
QBRS	1	eDNA	Loch Quoich	33.65	$8.87x10^{-6}$	$4.42x10^{-6}$
QBRS	2	eDNA	Loch Quoich	37.19	$6.88x10^{-7}$	$4.42x10^{-6}$
QBRS	3	eDNA	Loch Quoich	35.79	$1.89x10^{-6}$	$4.42x10^{-6}$
FSL1	1	eDNA	Loch Fearna	No Cq	0	0
FSL1	2	eDNA	Loch Fearna	36.23	$1.37x10^{-6}$	0
FSL1	3	eDNA	Loch Fearna	No Cq	0	0
FSL3	1	eDNA	Loch Fearna	No Cq	0	0
FSL3	2	eDNA	Loch Fearna	No Cq	0	0
FSL3	3	eDNA	Loch Fearna	36.26	$1.34x10^{-6}$	0
FSLX3	1	eDNA	Loch Fearna	No Cq	0	No Cq
FSLX3	2	eDNA	Loch Fearna	32.15	$2.62x10^{-5}$	$1.41x10^{-5}$
FSLX3	3	eDNA	Loch Fearna	34.14	$6.23x10^{-6}$	$1.41x10^{-5}$
FSL4	1	eDNA	Loch Fearna	32.50	$2.03x10^{-5}$	0
FSL4	2	eDNA	Loch Fearna	No Cq	0	0
FSL4	3	eDNA	Loch Fearna	No Cq	0	0
FSL5	1	eDNA	Loch Fearna	No Cq	0	0
FSL5	2	eDNA	Loch Fearna	34.68	$4.22x10^{-6}$	0
FSL5	3	eDNA	Loch Fearna	No Cq	0	0
FSL5B	1	eDNA	Loch Fearna	No Cq	0	0
FSL5B	2	eDNA	Loch Fearna	37.67	$4.87x10^{-7}$	$6.44x10^{-7}$
FSL5B	3	eDNA	Loch Fearna	36.21	$1.40x10^{-6}$	$6.44x10^{-7}$

Table 4: ***Results of qPCR assay amplifying the CO1 gene of S. Alpinus.*** *Cq and concentration values averaged from triplicate results in AriaMx software.*

Sample ID	Sample Type	Location	Cq	Quantity (ng)
NTC	Non-template control	N/A	35.52	$2.93x10^{-6}$
NTC	Non-template control	N/A	34.08	$8.14x10^{-6}$
1/10	Standard	Loch Ard Achadh	15.76	3.46
1/100	Standard	Loch Ard Achadh	18.42	0.346
1/1000	Standard	Loch Ard Achadh	22.36	0.0345
1/10,000	Standard	Loch Ard Achadh	25.69	0.00346
1/100,000	Standard	Loch Ard Achadh	29.08	0.000346
1/1,000,000	Standard	Loch Ard Achadh	32.96	$3.46x10^{-5}$
1/10,000,000	Standard	Loch Ard Achadh	34.26	$3.46x10^{-6}$
QLFT1	eDNA	Loch Quoich	No Cq	0
QUP1	eDNA	Loch Quoich	38.72	$3.06x10^{-7}$
QUP2	eDNA	Loch Quoich	35.09	$3.97x10^{-6}$
Q2R	eDNA	Loch Quoich	33.96	$8.84x10^{-6}$
QBRS	eDNA	Loch Quoich	34.84	$4.75x10^{-6}$
FSL1	eDNA	Loch Fearna	38.81	$2.89x10^{-7}$
FSL3	eDNA	Loch Fearna	38.1	$4.77x10^{-7}$
FSLX3	eDNA	Loch Fearna	33.53	$1.2x10^{-5}$
FSL4	eDNA	Loch Fearna	34.54	$5.87x10^{-6}$
FSL5	eDNA	Loch Fearna	36.45	$1.53x10^{-6}$
FSL5B	eDNA	Loch Fearna	37.44	$7.57x10^{-7}$